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A collaborative study to establish the 1st WHO International Standard for human cytomegalovirus for nucleic acid amplification technology

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ABSTRACT

Variability in the performance of nucleic acid amplification technology (NAT)-based assays presents a significant problem in the diagnosis and management of human cytomegalovirus (HCMV) infections. Here we describe a collaborative study to evaluate the suitability of candidate reference materials to harmonize HCMV viral load measurements in a wide range of NAT assays. Candidate materials comprised lyophilized Merlin virus, liquid Merlin virus, liquid AD169 virus, and purified HCMV Merlin DNA cloned into a bacterial artificial chromosome. Variability in the laboratory mean HCMV concentrations determined for virus samples across the different assays was 2 $\log_{10}$. Variability for the purified DNA sample was higher (>3 $\log_{10}$). The agreement between laboratories was markedly improved when the potencies of the liquid virus samples were expressed relative to the lyophilized virus candidate. In contrast, the agreement between laboratories for the purified DNA sample was not improved. Results indicated the suitability of the lyophilized Merlin virus preparation as the 1st WHO International Standard for HCMV for NAT. It was established in October 2010, with an assigned potency of 5×10^6 International Units (IU) (NIBSC code 09/162). It is intended to be used to calibrate secondary references, used in HCMV NAT assays, in IU.

1. Introduction

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus which causes disease in the immunologically-naïve, such as newborns and infants, and in immunosuppressed individuals, particularly transplant recipients and AIDS patients. Severe and life-threatening HCMV infections in immunocompromised individuals are managed with antiviral drugs, however, toxicity is associated with their prolonged use. The clinical utility of HCMV viral load measurements using nucleic acid amplification technology (NAT), for the diagnosis, prevention and treatment of disease in transplant recipients has been described [1]. Guidelines recommend using NAT to monitor HCMV load during pre-emptive antiviral therapy in order to guide the initiation of and determine the duration of treatment [2–4]. Antiviral drugs are deployed when pre-determined levels predictive of disease are reached. Although there is no consensus on the optimal sample type or frequency of testing, both plasma and whole blood provide pertinent information relevant to the diagnosis and prognosis of HCMV disease.

A wide variety of different NAT assays are used to determine HCMV viral load measurements. Many sites use laboratory-developed tests (LDTs) based on real-time PCR. A number of commercial assays are also available, comprising either analyte-specific reagents (ASR) or assay kits specific for different amplification platforms. Assays differ in the specimen tested (whole blood, plasma, serum, urine, CSF, etc), nucleic acid extraction protocol, PCR reagents (including primers and probes) and instrumentation used. Quantitative assays require control materials to determine the concentration of viral DNA present. These may comprise either a plasmid clone of the PCR target, or quantified viral DNA or virus particles, and depending upon the assay design, these may be excluded from the extraction step.

Given the heterogeneity of these NAT-based assay systems, and the lack of traceability to a standardized reference system, it is difficult to compare viral load measurements between different laboratories and to establish internationally applicable quantitative cut-off values for the diagnosis and treatment of CMV disease. Indeed, variability in the performance of different assays for HCMV

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has been documented [5,6]. These studies have highlighted the need for an internationally-accepted reference standard for HCMV. In 2004, the International Herpes Management Forum called for; 'an international quantification standard… to compare studies using different PCR-based systems and to facilitate patient management at multiple care centres' [2]. In the absence of such a standard, pre-existing clinical guidelines recommended that individual laboratories establish their own viral load cut-off values for HCMV management, which are specific to their laboratory assay [7].

The World Health Organization's (WHO) Expert Committee on Biological Standardization (ECBS) establishes reference standards for biological substances used in the prevention, treatment or diagnosis of human disease. WHO International Standards are recognized as the highest order of reference for biological substances, and are arbitrarily assigned a potency in International Units (IU). Their primary purpose is to calibrate secondary references used in routine laboratory assays, in terms of the IU, thereby providing a uniform result reporting system, and traceability of measurements, independent of the method used [8]. WHO International Standards for NAT for the blood-borne viruses have been shown to greatly improve the comparability of NAT assays used in blood safety and clinical fields [9].

Proposals for the development of the 1st WHO International Standard for HCMV were presented at the first SoGAT Clinical Diagnostics meeting held at NIBSC in June 2008 [10], and options for source materials and formulation of the candidate standards were discussed (http://www.nibsc.org/PDF/SoGAT%20Clinical%20Diagnostics%20I%202008%20Report.pdf). These proposals have now been realized through the development of candidate reference materials, evaluation of these in an international collaborative study, and establishment of the optimal candidate as the 1st WHO International Standard for HCMV for NAT.

2. Materials and methods

2.1. Preparation of candidate stock materials

Candidate materials comprised whole virus preparations of Merlin [11] and AD169 [12], a lyophilized whole virus Merlin preparation, and full-length Merlin DNA cloned into a bacterial artificial chromosome (BAC) [13]. Merlin and AD169 strains (kindly provided by Professor Wilkinson, Cardiff University, Cardiff, UK) were propagated in MRC-5 cells (NIBSC, Potters Bar, UK), in roller bottles. Tissue culture fluid was harvested once a cytopathic effect (CPE) was observed, and repeated until all the cells showed CPE. The culture supernatant was clarified by low speed centrifugation (3900× *g*) and the virus was pelleted by ultracentrifugation (48,000× *g*). Virus stocks were prepared by reconstituting virus pellets and pooling in a total volume of 200 mL of 10 mM Tris–HCl buffer (pH 7.4), containing 0.5% human serum albumin (HSA) (Tris–HSA buffer). The HSA used in the preparation of these candidate materials was derived from licensed products, and was screened and tested negative for anti-HIV-1, HBsAg, and HCV RNA. Virus stocks were stored at −70 °C until use. The Merlin BAC (pAL1128) (kindly provided by Professor Wilkinson, Cardiff University) had been prepared from the complete HCMV Merlin genome [13] (HCMV component sequenced, GenBank Accession number GU179001). BAC DNA was purified using a Nucleobond BAC100 kit (Machery-Nagel GmbH, Düren, Germany) according to manufacturer's instructions.

2.2. HCMV DNA quantification

The HCMV concentration of the Merlin and AD169 virus stocks was determined using a quantitative real-time PCR LDT (modified from Ref. [14]). Briefly, 400 μL of sample was extracted using the QIAamp® MinElute® Virus Spin Kit on the QIAcube® instrument (both QIAGEN, Hilden, Germany), and eluted into a final volume of 50 μL. Five microlitres of purified nucleic acid was then amplified by real-time PCR, using the LightCycler® 480 Instrument (Roche Applied Science, Mannheim, Germany). The target was quantified against serial dilutions of a plasmid clone of the PCR target. All samples were amplified in triplicate. The HCMV DNA concentration of the virus stocks was also determined using the LightCycler® CMV Quant Kit (Roche Diagnostics GmbH, Mannheim, Germany) and the Q-CMV Real Time Complete Kit (Nanogen Advanced Diagnostics S.r.L., Buttigliera Alta, Italy), and in five clinical laboratories in the UK using a range of LDT and commercial assays. The geometric mean virus concentration from all assays, in 'copies/mL', was used to determine a consensus HCMV concentration for each stock. The concentration of purified BAC DNA was determined by absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE).

2.3. Preparation of the candidate bulks

Merlin and AD169 whole virus candidate bulk materials were prepared by dilution of virus stocks in Tris-HSA buffer to a final concentration of 1×10^7 HCMV 'copies/mL'. Samples were dispensed in 1 mL aliquots and stored at −70 °C until shipment. The Merlin BAC bulk was prepared by dilution of the DNA stock in nuclease-free water to a final concentration of 1×10^5 HCMV 'copies/μL'. The rationale for preparing the Merlin BAC bulk at this concentration was to achieve broadly equivalent viral load determinations to the whole virus samples which were to be processed differently in the collaborative study evaluation (see Section on 2.7). The Merlin BAC bulk was aliquotted in 50 μL volumes and stored at −70 °C until shipment. The HCMV DNA concentration in each of these final study samples was determined using the real-time PCR LDT described above. The bulk for the lyophilized Merlin preparation, was formulated to contain approximately 1×10^7 HCMV 'copies/mL' in a final volume of 6.4 L Tris-HSA buffer. The bulk was mixed for a total of 30 min using a magnetic stirrer prior to filling.

2.4. Filling and lyophilization of the Merlin candidate

The filling of the lyophilized Merlin candidate was performed in a negative pressure isolator (Metall and Plastic GmbH, Radolfzell, Germany), containing the entire filling line, which is interfaced with a CS150 freeze dryer (Serail, Arguenteil, France). The bulk material was kept at 4 °C throughout the filling process and stirred constantly using a magnetic stirrer. The bulk was dispensed into 5 mL screw cap glass vials in 1 mL volumes, using a FVF5060 filling machine (Bausch & Strobel, Ilfshofen, Germany). The homogeneity of the fill was maintained by on-line check-weighing of the wet weight (target weight of 1.000 g), and vials outside the defined specification (0.993–1.008 g) were discarded.

Filled vials were partially stoppered with 14 mm diameter halobutyl cruciform closures and lyophilized in the CS150 freeze dryer. Vials were loaded onto the shelves at −50 °C and held at this temperature for 4 h. A vacuum was applied to 270 μb over 1 h followed by ramping to 30 μb over 1 h. The temperature was then raised to −40 °C and the vacuum maintained at this temperature for 42.5 h. The shelves were ramped to 25 °C over 15 h before releasing the vacuum and back-filling the vials with nitrogen. The vials were then stoppered in the dryer, removed and capped in the isolator, and the isolator decontaminated by fumigation with formaldehyde vapour before removal of the product. The sealed

vials are stored at −20 °C at NIBSC under continuous temperature monitoring for the lifetime of the product.

Assessments of residual moisture and oxygen content, as an indicator of vial integrity after sealing, were determined for twelve vials of freeze-dried product. Residual moisture was determined using a non-invasive MCT 600P Near Infrared (NIR) Transmitter (Process Sensors, Corby, UK). NIR results were then correlated to Karl Fischer (using calibration samples of the same excipient, measured using both NIR and Karl Fischer methods), to give % w/w moisture readings. Oxygen content was measured using a FMS-750 Oxygen Headspace Analyzer (Lighthouse Instruments, Charlottesville, VA).

2.5. Stability assessment of the lyophilized Merlin preparation

The stability of the lyophilized Merlin preparation is being assessed in an on-going accelerated thermal degradation study in order to predict the stability of the product when stored at the recommended temperature of −20 °C. Vials of the lyophilized product are being held at −70 °C, −20 °C, 4 °C, 20 °C, 37 °C, 45 °C. At specified time points during the life of the product three vials are removed from storage at each temperature and HCMV DNA quantified using the real-time PCR LDT described in Section 2.2.

2.6. Study participants

Thirty-two laboratories from 14 countries participated in the collaborative study, and are listed in the Acknowledgements. Participants were selected for their experience in HCMV NAT and geographic distribution. They represented mainly clinical laboratories, but also included a range of manufacturers of *in vitro* diagnostic devices (IVDs), as well as reference, research and quality assurance laboratories. All participating laboratories are referred to by a code number, allocated at random, and not representing the order of listing in the Acknowledgements. Where a laboratory returned data using different assay methods, the results were analyzed separately, as if from different laboratories, and are referred to as, for example, laboratory 1A, 1B etc.

2.7. Study design

Study samples were coded as samples 1–4 and were as follows; Sample 1, lyophilized Merlin virus; sample 2, liquid Merlin virus; sample 3, liquid AD169 virus; and sample 4, liquid preparation of purified BAC-cloned Merlin DNA. Four vials each of study samples 1–4 were delivered to participating laboratories by courier on dry ice with specific instructions for storage and reconstitution. Participants were requested to test dilutions of each sample using their routine HCMV NAT assay, on four separate occasions, using a fresh vial of each sample in each independent assay. The lyophilized sample 1 was to be reconstituted with 1 mL of deionized nuclease-free molecular-grade water and left for a minimum of 20 min with occasional agitation before use. Meanwhile, study samples 2–4 were to be thawed and vortexed briefly before use. For quantitative assays, participants were requested to test a minimum of two serial ten-fold dilutions within the linear range of the assay. For qualitative assays, participants were requested to test ten-fold serial dilutions of each sample in the first assay in order to determine the assay end-point. Then to fine tune the end-point by testing a minimum of two half-$\log_{10}$ serial dilutions either side of the predetermined end-point for subsequent assays. Participants were requested to dilute samples 1–3 using the sample matrix specific to their individual assay and to extract each dilution prior to amplification. Meanwhile, participants were requested to dilute sample 4 in nuclease-free water and add an aliquot of each dilution directly to the amplification reaction. Participants were requested to report the viral load in 'copies/mL' (positive/negative for qualitative assays) for each dilution of each sample, and to return results, including details of the methodology used, to NIBSC for analysis.

2.8. Statistical methods

Qualitative and quantitative assay results were evaluated separately. In the case of qualitative assays, for each laboratory and assay method, data from all assays were pooled to give a number positive out of a total number tested at each dilution step. A single 'end-point' for each dilution series was calculated, to give an estimate of 'NAT detectable units/mL', as described previously [15]. In the case of quantitative assays, analysis was based on the results reported by the participants in copies/mL. For each assay run, a single estimate of $\log_{10}$ 'copies/mL' was obtained for each sample, by taking the mean of the $\log_{10}$ estimates of 'copies/mL' across replicates, after correcting for any dilution factor. A single estimate for the laboratory and assay method was then calculated as the mean of the $\log_{10}$ estimates of 'copies/mL' across assay runs. Overall analysis was based on the $\log_{10}$ estimates of 'copies/mL' or 'NAT detectable units/mL'. Overall mean estimates were calculated as the means of all individual laboratories. Variation between laboratories (inter-laboratory) was expressed as the SD of the $\log_{10}$ estimates and percentage geometric coefficient of variation (%GCV) [16] of the actual estimates.

The ability of a candidate standard to reduce the inter-laboratory variability in HCMV viral load measurements was assessed by calculating the potency of one candidate relative to the other study samples. The relative potency of, for example, sample 3 relative to sample 1 was calculated for each individual assay as the difference in estimated $\log_{10}$ 'units per mL' (test sample − candidate standard) plus a candidate assigned value in IU/mL for the candidate standard.

Variation within laboratories and between assays (intra-laboratory), was expressed as the SDs of the $\log_{10}$ estimates and %GCVs of the individual assay mean estimates. These estimates were pooled across samples 1 to 3, but were calculated separately for sample 4. The significance of the inter-laboratory variation relative to the intra-laboratory variation was assessed by an analysis of variance.

3. Results

3.1. Validation of study samples and stability of the lyophilized candidate

Evaluation of multiple aliquots (n = 18) of each study sample prior to dispatch indicated that the homogeneity of HCMV content was similar for all study samples (2SD less than 0.3 $\log_{10}$ 'copies/mL' for each sample). The mean and CV of the fill weight for the lyophilized Merlin candidate, were determined from measurements from every 50th vial (n = 126), and were 1 g and 0.23%, respectively. The mean residual moisture, as determined by Karl Fischer and NIR, was 0.6% and 0.41%, respectively (CV = 7.2%), based on measurements from 12 vials. The mean residual oxygen content was 0.22% (CV = 40.6%), based on measurements from 12 vials. The CV of the fill mass and mean residual moisture were within acceptable limits for a WHO International Standard [8]. Residual oxygen content was within the NIBSC working limit of 1.1%.

The stability of the lyophilized Merlin candidate is being assessed in accelerated thermal degradation studies. Vials are stored at elevated temperatures and are removed at specific time points for testing using the HCMV real-time PCR LDT. The mean estimated HCMV concentration (in $\log_{10}$ 'copies/mL') for three vials

stored at each temperature for 4, 8 12, 29, 45 months, and 5 years are shown in Table 1. The differences in HCMV concentration from the −20 °C baseline sample are also shown. A negative value indicates a drop in potency relative to the −20 °C baseline. The 95% confidence intervals for the differences are ±0.16 $\log_{10}$ based on a pooled estimate of the SD between individual vial test results. The majority of temperatures and time points do not show any drop in potency compared with the −20 °C baseline samples. There is no observable change in potency across the course of the 5 years at any temperature, within the limits of the assay variability. However, there does appear to be a pattern of an apparent increase in potency with increasing temperature for the early time points. The reason for this is unclear.

3.2. Data received

Data were received from all 32 participating laboratories. Participants performed a variety of different assay methods with some laboratories performing more than one assay method. The variables in assay methodologies used are detailed in Table 2. In total, datasets were received from 53 quantitative assays and 5 qualitative assays. The majority of participants prepared dilutions of study samples 1–3 using either plasma (30 datasets) or whole blood (25 datasets), however, urine, PBS, and nuclease-free water were also used. The extent of the dilutions performed varied slightly between each laboratory. Extractions were predominantly automated. The majority of datasets reported the use of real-time PCR technology. Seventeen participants used commercial assays and reagents (37 datasets), while 13 participants used laboratory-developed assays (17 datasets). Two participants used both commercial and laboratory-developed assays (4 datasets). Given the range of assay combinations and variables, and the fact that no two assays were alike (apart from two laboratories using the Roche COBAS® AMPLICOR CMV MONITOR Test), it was not possible to group assays and perform the analysis according to the methodology used.

Laboratory 31 had anomalous results for sample 1 in assay 4 (negative at $10^{-4.5}$ to 10^{-6} but positive at $10^{-6.5}$ dilutions). The results for this assay were excluded from the analysis. Laboratories 2B, 4, 19B, 19C and 25 did not return results for sample 4. This was principally because, for these assays, it was not possible to determine viral load without extracting the sample. Laboratory 12A reported problems with their second assay for most replicates of samples 1, 2 & 3. This assay run was excluded from further analysis. Laboratory 16 only provided data from two assay runs. The second assay tested extracted material that had undergone one freeze/thaw cycle and was therefore excluded from the analysis. Laboratory 22B returned data from four assay runs. However, the last two runs tested samples that had undergone one freeze/thaw cycle and were therefore excluded from the analysis. For some laboratories and assays, the results from individual dilutions were excluded when they were noted as being outside the linear range of the assays. Apart from the cases noted above, there were no exclusions of data.

3.3. Potency estimates and inter-laboratory variation

The laboratory mean estimates for each study sample for quantitative assays (in $\log_{10}$ 'copies/mL') and qualitative assays (in $\log_{10}$ 'NAT detectable units/mL') are shown in Supplementary Tables 1 and 2, respectively. The individual laboratory mean estimates for each assay and study sample are also shown in histogram form in Fig. 1. Results for samples 1–3 show considerable variation in the viral load reported between different assays, with estimates differing by up to 2 $\log_{10}$ 'copies/mL' (100-fold) (Supplementary Table 1). The estimates from qualitative assays were typically lower than those for quantitative assays. Meanwhile, the variability for sample 4 was greater than that for samples 1–3, although, this was principally due to outlying results from five different assays (Fig. 1). Evaluation of the spread of results based on the dilution matrix used in each assay showed that there was no observed relationship between this and the HCMV concentration for each sample (data not shown).

Table 3 shows the overall mean estimates for each study sample, for quantitative and qualitative assays, along with the SD (of $\log_{10}$ estimates) and the %GCV (of actual estimates). For samples 1–3, the SD for quantitative assays is approximately 0.5 log, and the %GCV is approximately 200%. These figures are consistent with the observed 2 $\log_{10}$ 'copies/mL' range of estimates. The spread for the qualitative assays is similar. The SD and %GCV for sample 4 are higher than those for samples 1–3, again, most likely due to the outlying results.

Comparison of overall mean estimates for the lyophilized Merlin candidate sample 1 and liquid Merlin candidate sample 2 indicates that there was no significant loss in potency upon freeze-drying (Table 3). In addition, comparison of overall mean estimates for Merlin (sample 2) and AD169 (sample 3) liquid candidates indicates the suitability of all assays to quantify equally these two strains.

3.4. Relative potency determination

The expression of the potency of the study samples relative to the candidate standard (as described in Section 2.8), allows an assessment of the suitability of a candidate for the standardization of HCMV viral load measurements by NAT. The relative potencies of study samples 2–4 against candidate sample 1, for each quantitative and qualitative assay, are shown in histogram form in Fig. 2. Units are expressed as candidate $\log_{10}$ IU/mL. The overall mean relative potency estimates (in 'candidate $\log_{10}$ IU/mL') for samples 2–4, for quantitative and qualitative assays, along with the SD (of $\log_{10}$ estimates) and the %GCV (of actual estimates), are shown in Table 4. Fig. 2 and Table 4 show that when the mean estimates of samples 2 and 3 are expressed relative to sample 1, there is a marked improvement in agreement between laboratories, compared with Fig. 1 and Table 3. While the results from the qualitative assays remain more variable, they are now centred around the overall mean HCMV estimate. For the quantitative assays, the SD has reduced from approximately 0.5 $\log_{10}$ to 0.12

Table 1
Thermal stability of lyophilized HCMV (Merlin strain), 09/162, at different storage temperatures.

Temperature (°C)	Mean $\log_{10}$ 'copies/mL' (difference in $\log_{10}$ 'copies/mL' from −20 °C baseline sample)					
	4 months	8 months	12 months	29 months	45 months	60 months
−70	6.92	6.77	6.86	6.81	6.73	6.85
−20	6.92	6.78	6.85	6.62	6.70	6.80
4	6.86 (−0.06)	6.72 (−0.06)	6.77 (−0.08)	6.76 (0.14)	6.70 (0.00)	6.96 (0.16)
20	6.96 (0.04)	6.84 (0.06)	6.89 (0.04)	6.87 (0.25)	6.90 (0.20)	6.95 (0.15)
37	7.02 (0.10)	6.91 (0.13)	6.94 (0.09)	6.91 (0.29)	6.88 (0.18)	6.88 (0.08)
45	7.07 (0.15)	6.97 (0.19)	6.99 (0.14)	6.92 (0.30)	6.82 (0.12)	6.89 (0.09)

Table 2
Summary of assay methodologies.

Assay component	Variable
Diluent	Plasma, whole blood, urine, PBS, nuclease-free water.
Extraction (automated)	Abbott *m*2000sp; QIAGEN QIAsymphony SP and RG Q, BioRobot, MDx, and EZ1; bioMérieux NucliSENS® easyMag®; Roche MagNA Pure LC and COBAS® AmpliPrep; Siemens VERSANT® kPCR.
Extraction (manual)	Roche High Pure Viral Nucleic Acid Kit; Nanogen EXTRAgen®; QIAGEN QIAamp (Blood DNA, DNA and Viral RNA) Mini Kits, QIAGEN QIAamp DSP Virus Kit; Cepheid affigene® DNA Extraction Kit; phenol-chloroform extraction.
Amplification kit/ASR (commercial assays)	Roche COBAS® AMPLICOR CMV MONITOR Test; Nanogen Q-CMV Real Time Complete Kit; Argene CMV R-gene™ and CMV HHV6,7,8 R-gene™; QIAGEN *artus* CMV (LC and RG) PCR Kits; Roche COBAS® TaqMan® CMV Test; Cepheid affigene® CMV trender and SmartCMV™; Abbott RealTime CMV (in development in 2010); 'ELITech/Epoch CMV 3.0'; Quantification of CMV PrimerDesign™ Ltd.
HCMV gene target	*UL122*/*UL123* (immediate-early proteins), *UL54* (DNA polymerase), *UL83* (tegument protein pp65), *UL55* (envelope glycoprotein B), *US8* (membrane glycoprotein), *UL34* (nuclear egress membrane protein) and *UL80.5* (capsid assembly protein precursor).
Amplification platform	Roche LightCycler® 1.5, 2.0 and 480 systems, COBAS® TaqMan® and COBAS® AMPLICOR Analyzer; Applied Biosystems™ 7300, 7500, 7500 Fast, and 7900 HT Fast Real-Time PCR Systems; Agilent Mx3000P® qPCR System; QIAGEN Rotor-Gene Q, Rotor-Gene 3000 and 6000 instruments; Cepheid SmartCycler™ II; Bio-Rad MyCycler™.

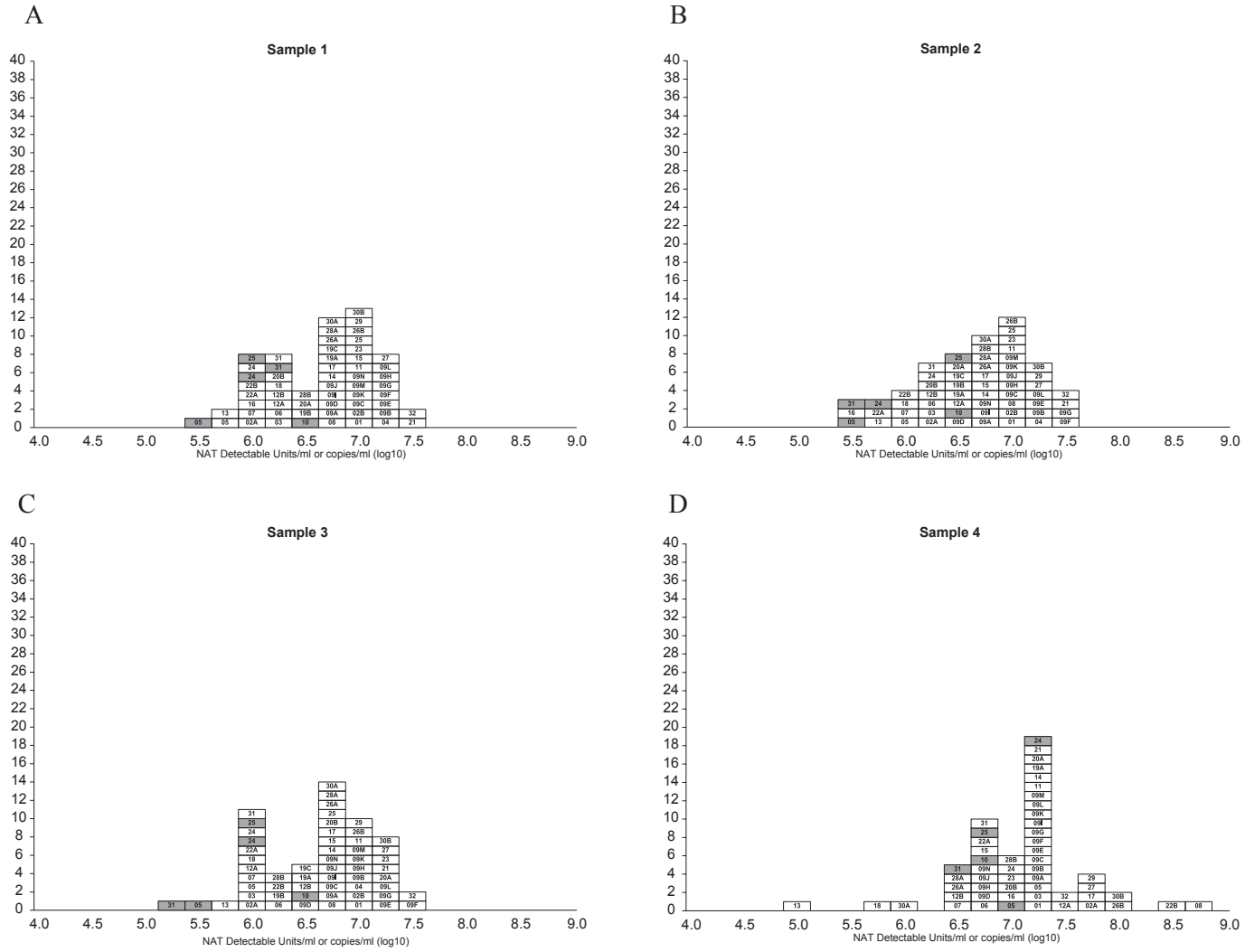

Fig. 1. Individual laboratory mean estimates for study samples 1 (A), 2 (B), 3 (C), and 4 (D), obtained using qualitative or quantitative NAT assays. Each box represents the mean estimate from each laboratory assay and is labelled with the laboratory code number. The results from the qualitative assays are shaded in grey.

$\log_{10}$ and 0.19 $\log_{10}$ for samples 2 and 3 respectively. However, when the mean estimates of sample 4 are expressed relative to sample 1 (Fig. 2), there is no significant improvement in agreement between laboratories, compared with Fig. 1. There is no reduction in the SD for quantitative assays. As sample 1 requires extraction, and sample 4 does not, differences in extraction efficiency between laboratories and methods still contribute to the observed variation between laboratories for sample 4. These results suggest that using sample 1 as an International Standard would significantly improve the agreement in HCMV estimates

Table 3
Overall mean estimates and inter-laboratory variation (log_{10} 'copies/mL' for quantitative or 'NAT-detectable units/mL' for qualitative assays).

Sample	Assay	No. of datasets	Mean	Range	SD	%GCV
1	qualitative	5	6.01	5.39–6.55	0.42	161
	quantitative	53	6.71	5.65–7.46	0.46	188
2	qualitative	5	5.93	5.47–6.43	0.46	185
	quantitative	53	6.71	5.58–7.53	0.49	207
3	qualitative	5	5.86	5.18–6.62	0.54	249
	quantitative	52	6.72	5.73–7.39	0.46	190
4	qualitative	5	6.82	6.51–7.16	0.25	77
	quantitative	48	7.11	5.06–8.81	0.61	307

between assays testing virus-based samples similar to study samples 2 and 3.

The estimated concentrations of samples 1–3 were also expressed in 'candidate IU', relative to sample 4, using a hypothetical unitage of 10^7 IU/mL for sample 4. The relative potencies of study samples 1–3 against candidate sample 4, for each quantitative and qualitative assay, are shown in histogram form in Fig. 3. The overall mean potency estimates in candidate log_{10} IU/mL, for samples 1–3 relative to sample 4, for quantitative and qualitative assays, are shown in Table 5. These results show that when the purified DNA sample 4 is used as a standard, there is no improvement in agreement between laboratories, as compared with Fig. 1. The SD between laboratories has increased from approximately 0.5 log_{10} to 0.64 log_{10}, while the %GCVs have increased to over 300%.

3.5. Intra-laboratory variation

Supplementary Table 3 shows the intra-laboratory SDs and % GCVs for each laboratory, calculated by pooling the HCMV viral load estimates for samples 1–3, but separately for sample 4. For all samples, the inter-laboratory variation was greater than the intra-laboratory variation ($p < 0.0001$). For samples 1–3, there were differences between the repeatability of laboratory estimates across assays (mean SD of 0.11 log_{10}, mean %GCV of 30%). For

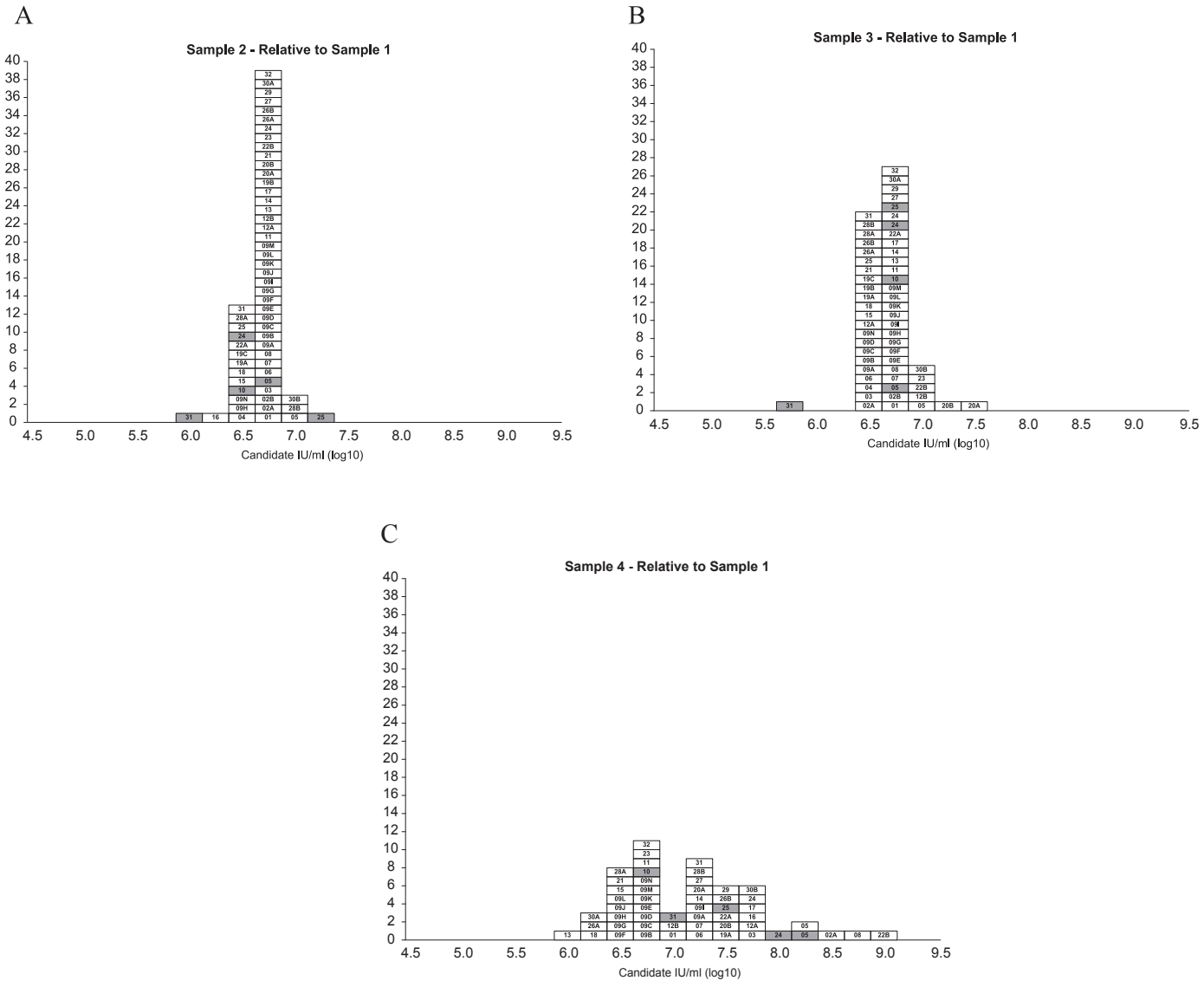

Fig. 2. Potencies of sample 2 relative to sample 1 (A), sample 3 relative to sample 1 (B), and sample 4 relative to sample 1(C), for each quantitative and qualitative assay. Units are expressed as candidate log_{10} IU/mL in both cases. Each box represents the relative potency for each laboratory assay and is labelled with the laboratory code number. The results from the qualitative assays are shaded in grey.

Table 4
Overall mean estimates and inter-laboratory variation for potency (log_{10} IU/mL) relative to sample 1, taking sample 1 as 5×10^6 (6.7 log_{10}) IU/mL.

Sample	Assay	No. of datasets	Mean	Range	SD	%GCV
2	qualitative	5	6.62	6.02–7.16	0.42	163
	quantitative	53	6.70	6.31–6.90	0.12	31
	combined	58	6.69	6.02–7.16	0.16	44
3	qualitative	5	6.56	5.73–6.84	0.47	192
	quantitative	52	6.69	6.42–7.46	0.19	56
	combined	57	6.68	5.73–7.46	0.23	68
4	qualitative	5	7.52	6.83–8.23	0.58	280
	quantitative	48	7.12	6.11–8.99	0.64	341
	combined	53	7.16	6.11–8.99	0.64	340

Table 5
Overall mean estimates and inter-laboratory variation for potency (log_{10} IU/mL) relative to sample 4, taking sample 4 as 10^7 (7.0 log_{10}) IU/mL.

Sample	Assay	No. of datasets	Mean	Range	SD	%GCV
1	qualitative	5	6.18	5.47–6.87	0.58	280
	quantitative	48	6.58	4.71–7.59	0.64	341
	combined	53	6.54	4.71–7.59	0.64	340
2	qualitative	5	6.10	5.61–6.70	0.51	221
	quantitative	48	6.59	4.70–7.73	0.64	333
	combined	53	6.54	4.70–7.73	0.64	334
3	qualitative	5	6.04	5.61–6.94	0.54	246
	quantitative	47	6.60	4.97–7.68	0.63	325
	combined	52	6.54	4.97–7.68	0.64	334

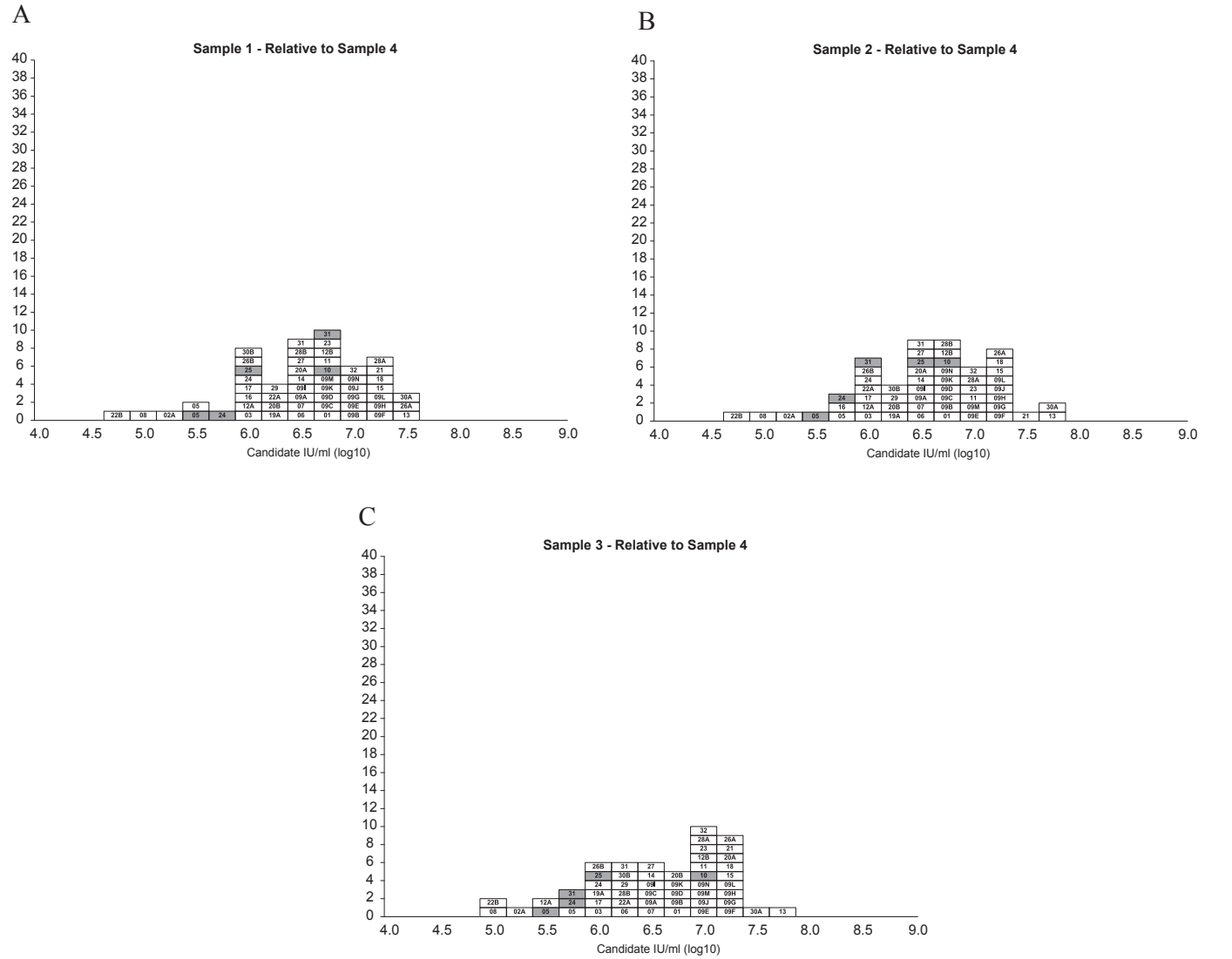

Fig. 3. Estimated concentrations (expressed in IU) of sample 1 relative to sample 4 (A), sample 2 relative to sample 4 (B), and sample 3 relative to sample 4 (C), using a hypothetical unitage of 10^7 IU/mL for sample 4. Each box represents the relative potency for each laboratory assay and is labelled with the laboratory code number. The results from the qualitative assays are shaded in grey.

sample 4, the intra-laboratory variability was greater (mean SD of 0.21 log_{10}, mean %GCV of 63%).

4. Discussion

The clinical management of an increasing number of infectious diseases is dependent on molecular detection and quantification methods in order to guide treatment. Viral load measurements need to be accurate and comparable across the range of assays in use in order to establish optimal treatment strategies. In the past, NIBSC has collaborated with the WHO ECBS in supporting the introduction of NAT assays into the blood safety field, through the development of International Standards and other reference materials for blood-borne viruses. These materials have helped to

standardize assays and improve the quality of data produced. Here we describe work to apply these same principles to support the need to standardize NAT assays used in the molecular diagnosis of a clinical virus, namely HCMV. The aim was to make a large batch of aliquots of a stable and homogeneous preparation suitable for a range of NAT-based assays.

A number of candidate materials were prepared and evaluated for their suitability as a HCMV International Standard. These included whole virus (liquid) preparations of Merlin and AD169 strains, purified Merlin DNA cloned into a BAC and a lyophilized whole virus preparation of the Merlin strain. Merlin and AD169 were selected for the study as they represent the prototype clinical and laboratory HCMV strains respectively. Merlin is a low-passage strain with a near-complete genome that has been fully sequenced (GenBank Accession number AY446894). Meanwhile, AD169 contains several deletions (up to 15 kb in size), compared with wild-type isolates. As the prototype laboratory strain, AD169 DNA is frequently used as a calibrator in HCMV NAT. Merlin and AD169 also represent different genotypes of HCMV, classified as genotype 1 and 2 viruses, respectively, based on the glycoprotein B gene.

The formulation of whole virus preparations enables the candidates to be extracted alongside assay controls and clinical samples, thereby standardizing the entire HCMV assay, including both extraction and DNA amplification steps. Compared with the blood-borne viruses, where virus is principally detected in one sample matrix (i.e. plasma), the design of a reference material for a clinical virus such as HCMV is particularly challenging, because of the number of substrates in which the virus is detected. Given the wide range of samples that are routinely tested for HCMV, and the different extraction methods designed to process each sample type, the whole virus candidates were formulated in a universal Tris-HSA buffer, to allow for further dilution in the appropriate sample matrix used in each laboratory assay. The intention here was to provide a single reference material for HCMV that could be processed using all extraction protocols available for HCMV-positive samples. A preparation of Merlin DNA cloned into a BAC was included in the study, in order to investigate the effect of the extraction step on the variability in HCMV quantification, and to determine whether a purified DNA sample is suitable for standardizing HCMV viral load measurements.

A lyophilized Merlin whole virus candidate was prepared, to enable shipping at ambient temperatures, and for long-term stability. Data from accelerated thermal degradation studies did not show any evidence for degradation after storage at elevated temperatures for 5 years. As there is no observed drop in potency, it was neither possible to fit the usual Arrhenius model for accelerated degradation studies, nor to obtain any predictions for the expected loss per year with long-term storage at −20 °C. However, using the 'rule of thumb' that the decay rate will approximately double with every 10 K increase in temperature, and noting that there is no detectable drop in potency after 60 months at 20 °C, then there should be no detectable difference after 480 months (40 years) at −20 °C. A similar argument applied to the 37 °C data would imply no detectable loss after more than 100 years at −20 °C. Overall, the available data indicates adequate stability for the lifetime of the standard which is expected to be 10–15 years. All of the candidate preparations were determined to be homogeneous in terms of the HCMV DNA content by PCR.

In the collaborative study, all assays detected both Merlin and AD169 strains, demonstrating the suitability of these HCMV strains for use as candidate reference materials. The overall mean estimates for the Merlin and AD169 whole virus candidates (samples 1–3) were ~5×10^6 (6.7 $\log_{10}$) 'copies/mL'. This is slightly lower than the original target concentration of 1×10^7 'copies/mL' and is likely to be due to the small subset of laboratories selected for preliminary testing of the virus stocks, and the large inter-laboratory variation observed in assay results. The overall range in laboratory mean estimates for the whole virus study samples 1–3 was 2 $\log_{10}$ 'copies/mL'. This variability reflects the range and differences in diagnostic testing procedures between laboratories and is similar to levels previously reported for HCMV NAT-based assays [5,6]. The overall range in laboratory mean estimates for the purified DNA sample 4 was higher. This was unexpected, since the purified DNA sample 4 was not extracted. However, the spread was principally due to outlying results from five assays (there was no observed relationship between these five assays). Inter-laboratory variability was significantly greater than intra-laboratory variability. This was also reported by Pang et al., [5].

The agreement between laboratories for virus samples 2 and 3 was markedly improved when the potencies of these study samples were expressed relative to the lyophilized Merlin candidate (sample 1). These results demonstrate the suitability of the virus-based candidates to reduce the inter-laboratory variability of assays testing similar whole virus samples, and confirms the ability of the Merlin candidate to calibrate secondary references comprising the AD169 strain. When the purified Merlin BAC preparation, sample 4, was used as the reference, it did not improve the agreement in HCMV quantification between laboratories. These results highlight the extent to which the extraction step contributes to inter-laboratory variability. They also imply that a reference standard based on DNA would not be suitable for the standardization of NAT assays testing whole virus samples.

From the results of this collaborative study, the lyophilized Merlin whole virus preparation was determined to be the optimal candidate for a higher order reference for HCMV quantification by NAT. This candidate was established at the WHO ECBS meeting in October 2010 as the 1st WHO International Standard for HCMV for NAT-based assays, with an assigned potency of 5×10^6 International Units, when reconstituted in 1 mL of nuclease-free water (NIBSC product code 09/162) [17]. The assignment of a unitage for a 1st WHO International Standard is arbitrary. However, in the case of this study, a value of 5×10^6 IU was chosen as this represents the consensus estimate for the candidate standard across all laboratory assays. The assigned unitage does not carry an uncertainty associated with it's calibration. The uncertainty may therefore be considered to be the variance of the vial content and was determined to be ±0.23%. As this study shows, the use of the term copy number for virus quantification by NAT can be misleading. Copy number estimates are not necessarily equivalent to genuine genome equivalent numbers, but are instead dependent upon variables in the extraction and amplification steps, and on the quantification controls used. As for the International Standards established for the blood-borne viruses, there is no overall conversion factor between copies and IUs for HCMV. Any conversion is entirely restricted to an individual assay, and the calibration of this assay to the International Standard. The HCMV International Standard is intended to be used for the calibration of whole virus-based secondary reference materials used in HCMV NAT assays. This can be performed by assaying serial dilutions of both preparations in parallel, and determining the equivalent concentration of the secondary reference in IU. Once reconstituted, the HCMV International Standard should be diluted in the matrix appropriate to the material being calibrated, and extracted and amplified in parallel with the secondary reference. For example, if the secondary reference comprises whole virus in a plasma matrix, then it should be tested in parallel with dilutions of the WHO International Standard prepared in a plasma matrix. The stability of the material when reconstituted has not been specifically determined. Therefore, it is recommended that the standard is for single use only.

Updated international consensus guidelines on the management of HCMV in solid-organ transplantation recommend that HCMV assays are recalibrated and demonstrate colinearity to the WHO International Standard, and that results are reported as IU/mL [4]. This study highlights the improved inter-laboratory agreement and comparability of viral load data when assays are calibrated against a common reference. It is hoped that in due course, harmonization of HCMV measurements will allow for clinically relevant thresholds for HCMV to be defined in IU [3,4,18]. This would negate the need for individual laboratories to establish their own cut-offs for HCMV management, and would allow patients to be managed at multiple care centres.

The ability of the lyophilized Merlin candidate to reduce inter-laboratory variability in HCMV quantification for the samples investigated in this study, does not guarantee that, when widely implemented, the HCMV International Standard will harmonize HCMV assays in the same way. The accuracy of measurement of a virus in a clinical sample relies on the ability of the reference or control samples to behave like clinical samples, i.e. to demonstrate commutability. According to ISO guidelines [19], commutability needs to be demonstrated for all references used in the measurement process, i.e. secondary and tertiary references and the higher order reference against which they are calibrated. Commutability can be affected by a range of factors including matrix and molecular variants of the analyte (in this case HCMV DNA). The commutability of the lyophilized Merlin candidate for HCMV-positive clinical samples was not specifically assessed in this study. However, the choice of strain and formulation of this candidate was intended to make it as similar as possible to the range of samples being investigated for HCMV. It was not feasible to derive the candidate bulk from HCMV-positive clinical material, as has been the case for other WHO International Standards such as those for HAV, HBV and HCV [20–22]. The purpose of formulating the candidate in a universal matrix was to try to control for matrix effects, as it allows for subsequent dilution in the sample matrix appropriate to each assay. In this study, the improved agreement of all assay results for samples 2 and 3 when expressed relative to the candidate standard was independent of the diluent matrix used. The lyophilized Merlin candidate was derived from a crude cell-free preparation of HCMV from cell culture, which comprises both whole virus and naked HCMV DNA forms (as determined by DNase digestion experiments – data not shown). Meanwhile, patient samples derived from peripheral blood are likely to comprise a range of HCMV forms including whole and disrupted virions, and fragmented genomic DNA, with different forms predominating in different blood compartments. Plasma and serum samples from renal transplant recipients have been reported to contain highly fragmented HCMV DNA [23]. Meanwhile, whole blood samples from the same patients comprised a mixture of highly fragmented and large DNA forms, some of which may have been derived from whole virus.

A full assessment of commutability would require the reference material to be evaluated in all assays and alongside all sample types for which it might be used. This is because commutability can only be demonstrated for the assays and clinical samples for which the reference has been assessed. This task is particularly challenging for HCMV because of the number of assays in use (as highlighted in this collaborative study), and also because of the range of sample types that are tested. The difficulty in sourcing sufficient volumes of clinical material for such extensive studies would require pooling or dilution of samples thereby potentially compromising matrix effects. This difficulty means that commutability will most likely need to be addressed in multiple studies.

Commutability of the HCMV International Standard with HCMV-positive plasma samples has recently been evaluated [24]. In this study, forty HCMV-positive plasma samples (pooled serially collected samples from individual solid organ transplant patients), were tested alongside dilutions of the HCMV International Standard by six laboratories performing six commercial and two LDT assays. The data was analyzed using linear regression and correspondence analysis approaches. The results showed that several of the assays (or assay pairs) showed either reduced commutability or noncommutability. While these assays comprise many different reagents including assay-specific secondary standards, in this study, it was not possible to determine what impact these variables had on the observed altered commutability between different assays. Further studies are planned to investigate the commutability of the HCMV International Standard with other HCMV-positive sample matrices. In the meantime, the availability of the HCMV International Standard provides a higher order reference, which has been demonstrated in this present study, to be homogeneous and stable, and to reduce the inter-laboratory variability for the assays and samples assessed in this study. Whether this improvement in the agreement for HCMV measurements will be seen for all NAT assays will be reported in time. Any evidence for non-commutability might restrict its use with specific assays or sample types and will be noted in the instructions for use that accompany the standard.

In addition to ongoing commutability studies for the HCMV International Standard, NIBSC is collaborating with the WHO ECBS to support the standardization of NAT assays for other clinically-important viruses. The 1st WHO International Standard for Epstein–Barr virus for NAT was established by the WHO ECBS in October 2011 [25]. Similar projects are underway to establish International Standards for BK virus, JC virus, adenovirus and human herpesvirus 6. It is anticipated that the future availability of an increasing number of higher order reference materials for clinically-important viruses will improve the standardization of viral load measurement and lead to better patient management.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biologicals.2016.04.005.

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